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(71) Applicant *(for all Designated States except the US)*: ASULAB S.A. [CH/CH]; Faubourg du Lac 6, CH-2501 Bienne (CH).

(72) Inventors; and

(75) Inventors/ Applicants *(US only)*: GRÄTZEL, Michael [DE/CH]; Chemin du Marquisat 7A, CH-1025 St-Sulpice (CH). FRASER, David [GB/CH]; Chemin des Cyprès 4, CH-1800 Vevey (CH). ZAKEERUDDIN, Shaik, Mohammed [IN/CH]; Rue du Lac 25B, CH-1020 Renens (CH). RANDIN, Jean-Paul [CH/CH]; Potat-Dessus 13, CH-2016 Cortaillod (CH). FRENKEL, Erik, Jan [NL/CH]; Rue du Vully 29, CH-2000 Neuchâtel (CH).

(74) Agent: I C B: Ingénieurs Conseils en Brevets SA, Passage Max-Meuron 6, CH-2001 Neuchâtel (CH)

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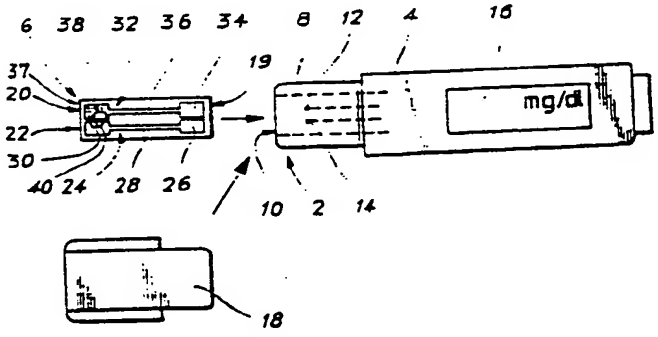
(54) Title: SENSOR FOR MEASURING THE QUANTITY OF A COMPONENT IN SOLUTION

(57) Abstract

The present invention relates to a sensor for measuring the quantity of a component in solution. The object of the invention is to improve the existing amperometric sensors. This object is achieved by means of a sensor provided with a measuring electrode (20) having at least one current collector (37), connected electrically to one of the electrical contacts (34) and covered by a mixture (38) containing at least one oxidation-reduction enzyme that is specific for the said component and at least one mediator that transfers the electrons between the said enzyme and the said current collector, characterized in that the mediator is chosen from among the complexes of a transition metal with at least one bipyridine, terpyridine or phenanthroline ligand substituted by at least one electron donor group. This sensor is applicable more particularly to the detection of glucose.



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# SENSOR FOR MEASURING THE QUANTITY OF A COMPONENT IN SOLUTION

The present invention relates to a sensor for measuring the quantity of a component in solution, which sensor is intended to be used in a device for amperometric measurement of the concentration of the said component in the solution. In particular, this sensor is capable of determining glucose.

Numerous patients suffering from diabetes must make frequent measurements of their blood glucose level or glycemia. If they detect a hyperglycemic condition, they must immediately take drugs to regulate their glucose level. To ease the everyday life of these patients, numerous miniaturized glucose-measuring devices that can be used by a neophyte have appeared on the market.

The implantation of insulin pumps in diabetics has also been envisioned. These insulin pumps must be equipped with glucose-measuring devices that are also implantable and that provide the pump with information for possible operation thereof as a function of the measured glycemia.

The majority of these devices for measuring glycemia use a glucose-specific enzyme known as glucose oxidase (GOD).

As illustrated in the attached Figure 1, GOD is a flavoprotein (obtained from molds, for example), which catalyzes the oxidation of glucose, in this case, for example, blood glucose, to gluconolactone, with formation of hydrogen peroxide ($H_2O_2$), from molecular oxygen ($O_2$) present in the solution to be tested, in this case blood.

This enzyme (GOD) and oxygen have therefore been used frequently in glucose-measuring devices in which oxidation of the glucose was detected by an electrical or optical transducer.

Similarly, this enzyme (GOD) and oxygen have often been used in so-called amperometric devices, and their application is described in the literature.

These so-called "amperometric" devices comprise on the one hand a measuring apparatus equipped with at least two electrical contacts

connected to an ammeter and to indicating means, and on the other hand a sensor, which may or may not be disposable, which can be connected to these two electrical contacts. This sensor has at least two electrodes, one a reference and the other the measuring electrode. The measuring electrode comprises a metal conductor covered with an enzyme that is specific for the product to be detected.

The attached Figure 2 illustrates the chemical reactions that take place at this measuring electrode. When the solution to be tested is deposited on the measuring electrode, the product to be tested (in this case glucose) reacts with the enzyme (in this case the oxidized GOD) present on the electrode to form gluconolactone, during which the GOD is converted to the reduced state ($GOD(H_2)(red)$). This reduced GOD then reacts with oxygen ($O_2$), which is converted to the reduced state ($H_2O_2$) and which then transfers two electrons ($e^-$) to the electrical conductor C, the potential of which is fixed at approximately 650 mV. The fact that it is necessary to work at high potentials causes additional problems of interferences. Oxygen therefore has a mediator role, since it permits the transfer of electrons. This transfer of electrons, which is proportional to the quantity of glucose present in the solution to be tested, is then measured by the ammeter, and the quantity of glucose present in the solution is indicated by the indicating means of the measuring apparatus.

Additional research has shown that amperometric devices using organic, inorganic or organometallic nonphysiological mediators could replace the devices that use oxygen as mediator. In fact, as can be seen from Figure 2, the devices that use oxygen as mediator cannot be used in solutions in which the stoichiometric concentration of oxygen is lower than the concentration of the component to be measured. Otherwise, although in this case the total quantity of the component to be measured has the opportunity to react with the oxidized enzyme to form the reduced enzyme, only part of the total quantity of reduced enzyme can react with the oxygen that is present, this part being proportional to the quantity of oxygen. The rest of the reduced enzyme cannot react, and the quantity of electrons transmitted to the conductor C is smaller than it should be.

Consequently, when this type of device is used, either a limit is imposed by the respective concentrations of oxygen and of the component to be measured, or it is necessary to use a membrane to limit the diffusion of the said component. This explains the attempts to develop amperometric devices that use a particular mediator other than oxygen.

A very large number of mediators have been cited in the literature, for example the monomeric ferrocenes (Cass, A.E.G. et al. (1984), Anal. Chem. 56, 667-671; Degani, Y. and Heller, A. (1987), J. Phys. Chem. 91, 1285-1289), the ferrocenes grafted onto a polymer (Foulds, N.C. and Lowe, C.R. (1988), Anal. Chem. 60, 2473-2478), the conductive charge-transfer salts (Alberry, W.J., Bartlett, P.N. and Craston, D.H. (1985), J. Electroanal. Chem. Interfacial Electrochem. 194, 223-235), the cyclame nickels (Taniguchi, I., Matsushita, K., Okamoto, M., Collin, J-P and Sauvage, J-P (1990), J. Electroanal. Chem. Interfacial. Electrochem. 280, 221-226) and organic compounds such as the quinones and the benzoquinones (Kulys, J.J. and Cénas, N.K. (1983), Biochim. Biophys. Acta 744, 57). Because of the important studies of Hill and his collaborators (for example, Frew, J.E. and Hill, H.A.O. (1987) (Phil. Trans. R. Soc. Lond. B316, 95-106), the family of ferrocene compounds has been widely established and used as mediator for GOD and other flavoproteins. Consequently, a commercially available sensor is now known that uses a member of the family of ferrocene compounds as mediator.

Unfortunately, the mediators existing at present rarely have the required ideal properties, or in other words electrochemical potential adapted to the chosen enzyme, adequate solubility, good chemical stability to light, heat and pH, and rapid interaction with the chosen enzyme.

In addition, the oxygen that may be present in the solutions to be tested competes with certain mediators according to the mechanism of the attached Figure 3. In fact, although the mediator (Med) present on the conductor C continues to react with some molecules of reduced GOD, it is possible that a certain quantity of the oxygen ($O_2$) that may be present will also react with other molecules of reduced GOD with formation of $H_2O_2$, as seen in the foregoing with reference to Figure 2. When measurements are made with a low potential between the measuring electrode and the reference electrode, the $H_2O_2$ traps the electrons produced by the

reaction between GOD and oxygen, and these electrons no longer migrate to the electrode. Since the quantity of oxygen in solution may vary, the quantity of trapped electrons also varies. Consequently, a proportional relationship no longer exists between the quantity of electrons migrating to the electrode and the quantity of glucose present in the solution to be tested. Under these conditions, therefore, such sensors do not yield reliable results.

The object of the invention is to remedy the disadvantages cited in the foregoing.

To this end, the invention relates to a sensor for measuring the quantity of a component in solution, comprising:

- at least one measuring electrode and one reference electrode, electrically insulated from each other and intended to come into contact with the said solution, the said electrodes respectively containing electrical contacts adapted to be connected to a device for processing the signal supplied by the said sensor,
- the measuring electrode containing at least one current collector, connected electrically to one of the said electrical contacts and covered with a mixture containing at least one oxidation-reduction enzyme that is specific for the said component and at least one mediator that transfers the electrons between the said enzyme and the said current collector.

According to the characteristics of the invention, the mediator is chosen from among the complexes of a transition metal with at least one bipyridine, terpyridine or phenanthroline ligand substituted by at least one electron donor group.

By virtue of the characteristics of the sensor according to the invention, and in particular by virtue of the new mediators being used, a family of sensors is obtained that have a broad range of low oxidation-reduction potentials, remain stable in air and provide a faster response than the other sensors of the prior art.

The invention will be better understood by reading the following description of the preferred embodiments of the invention, which are given by way of indication but are not limitative, this description being provided in connection with the attached drawings, wherein:

- Figure 1 illustrates the degradation of glucose in the presence of glucose oxidase (GOD),
- Figures 2 and 3 are mechanisms illustrating the various chemical reactions occurring at the sensors,
- Figure 4 is an overhead view of a measuring apparatus equipped with a sensor according to the invention,
- Figure 5 represents the cyclic voltametry curves of the tris(4,4'-dimethoxy-2,2'-bipyridine) complex of osmium in the absence of GOD and glucose, at different scan rates,
- Figure 6 represents substantially the same curves as Figure 5, but in the presence of GOD and glucose,
- Figure 7 represents three curves illustrating the variation of current density obtained after 30 seconds ($D_{30}$) as a function of glucose concentration in a physiological solution, for measurements performed with three types of sensors according to the invention, in which the quantity of carbon powder varies,
- Figure 8 represents the slope and ordinate at the origin of the curves of Figure 7, as a function of the quantity of carbon powder,
- Figure 9 represents three curves illustrating the variation of current density obtained after 30 seconds ($D_{30}$) as a function of glucose concentration in a physiological solution, for measurements performed with three types of sensors according to the invention, in which the quantity of glucose oxidase varies,
- Figure 10 represents the slope and ordinate at the origin of the curves of Figure 9, as a function of the quantity of glucose oxidase,
- Figure 11 represents three curves illustrating the variation of current density obtained after 30 seconds ($D_{30}$) as a function of glucose concentration in a physiological solution, for measurements performed with three types of sensors according to the invention, in which the quantity of mediator varies,
- Figure 12 represents the slope and ordinate at the origin of the curves of Figure 11, as a function of the quantity of mediator,

- Figure 13 represents measurements of the current density obtained as a function of glucose concentration, these measurements being performed in blood and in a phosphate buffer with glucose sensors provided respectively with one of the two preferred mediators according to the invention,

- Figure 14 represents measurements of the current density obtained as a function of glucose concentration, these measurements being performed with sensors according to the invention in blood samples having different hematocrits,

- Figure 15 and 16 represent measurements of the current density obtained as a function of glucose concentration, these measurements being performed with sensors according to the invention in physiological solution samples respectively having various concentrations of acetaminophenol and ascorbic acid.

As illustrated in Figure 4, the apparatus 2 for measuring the quantity of a given component in solution comprises a sensor 6 according to the invention and a device 4 for processing the signal provided by the said sensor. This device 4 is known in itself and has the general form of a pen. It is quite evident that this form is not limitative for the invention.

At one of its ends 8, this pen 4 has a cavity 10 in which there are housed two first electrical contacts 12, 14, which are connected electrically to an ammeter (not shown). This ammeter is in turn connected to an indicator 16, which indicates the concentration of the component being tested in a given solution. This concentration is indicated in mg/dl or in mmol/liter, for example. The pen 4 also has a stopper 1 [sic; 18 ?], which caps its end 8 and protects the contacts 12, 14 when the said pen is not being used.

The sensor 6 according to the invention has, for example, the form of an insulating rectangular lamella, which can be introduced by one of its ends 19 into the cavity 10 of the pen 4. It will be noted that this sensor 6 is provided for one-time use.

It comprises a measuring electrode 20 and a reference electrode 22 which, for example, are disposed longitudinally parallel on the sensor 6. The reference electrode 22 has a strip 24 of electrically conductive

material. This strip 24 has three zones, a zone 26 called the electrical contact, provided toward the end 19 of the said sensor, a central zone 28 called the "conducting track" and a zone 30, provided at the other end of the sensor and called the "current collector". In very similar manner, the measuring electrode 20 has a strip 32 of electrically conductive material 32. This strip 32 also has three zones, an electrical contact 34, a conducting track 36 and a current collector 37 which, in contrast to the collector 30, is covered with a mixture 38.

This collector 37 is not truly visible in Figure 4, since it is hidden by the mixture 38. In each of these electrodes, it will be noted that the collector and the current conductor could be electrically interconnected in two parts, and do not necessarily have to be in the form of a single strip 24 or 32. The mixture 38 contains at least one oxidation-reduction enzyme that is specific for the component to be measured and at least one mediator that transfer the electrons between the said enzyme and the current collector formed in the strip 32.

In an optimum embodiment, the aforesaid mixture 38 can also contain at least one active conductive material and/or at least one additive to be described later. If the mixture 38 contains an active conductive material, the mediator transfers the electrons between the enzyme and this active conductive material, which in turn transfers the electrons to the current collector.

The drop 40 of the sample of solution to be tested is deposited such that it straddles the two electrodes 20 and 22 as illustrated in Figure 4. Thus the electrical circuit consisting of the ammeter, the contacts 14 and 26, the conducting track 28, the collector 30, the solution drop 40, the mixture 38, the collector 37, the conducting track 36 and the contacts 34 and 12 is closed.

The measuring apparatus 2 just described is intended for carrying out measurements in vitro, but nevertheless it is quite evident that the sensor 6 could be used in vivo, in implantable measuring apparatuses. In this case, its form or its dimensions would be adapted to this new application.

In addition, to obtain increased precision, there could be added a second measuring electrode, identical to the measuring electrode 20 but without the enzyme or with the denatured enzyme.

The drop 40 of solution to be tested can be of biological nature, for example, blood or urine of a human being or of an animal, or a medium for fermentation of microorganisms. It may be of synthetic origin, for example a synthetic buffer containing the elements to be analyzed.

As oxidation-reduction enzyme there is used an enzyme specific for the component to be measured. According to the invention, it is preferable to use an enzyme chosen from among the oxidases and the flavoproteins. When it is wished to construct a glucose sensor, glucose oxidase (GOD), is used, for example a GOD having an activity of about 250 IU, obtained from an Aspergillus niger mold.

The optional active conductive material is preferably used in the form of powdered carbon, graphite, gold, platinum, palladium or conductive metal oxide, for example ruthenium oxide, or in the form of a conductive polymer firm, for example polypyrrole. Carbon powder will be used preferably.

As seen in the foregoing, there can also be included an additive that forms a network for immobilization of the enzyme, of the mediator and/or of the active conductive material on the surface of the collector 37 of the measuring electrode 20. This additive is, for example, bovine serum albumin (BSA), glutaraldehyde, carbodiimide or water-soluble polymers.

The electrically conductive material strips 24, 32 are made, for example, in the form of a film of material chosen from among gold, silver, platinum, palladium, carbon, graphite or a conductive metal oxide, such as a ruthenium oxide, for example. Preferably, the strip 24 corresponding to the reference electrode 22 is made of silver, and the strip 32 corresponding to the measuring electrode 20 is made of platinum. More precisely, the part of the strip 24 corresponding to the current collector 30 is partly converted to chloride.

It has been discovered that a new family of complexes of a transition metal with at least one bipyridine, terpyridine or phenanthroline ligand substituted by at least one electron donor group exhibits good mediator properties.

Preferably the electron donor group is an OH group, an alkoxy group, an aryloxy group or a primary, secondary or tertiary amine group.

In the case of a glucose sensor, and when glucose oxidase (GOD) is used as enzyme, the tris(4,4'-dimethoxy-2,2'-bipyridine) complex of osmium or the bis(4,4'-dimethoxy-2,2'-bipyridine)-mono-(4,4'-dimethyl-2,2'-bipyridine) complex of osmium is preferably chosen from among the aforesaid mediators.

In the case of a glucose sensor, the mixture 38 deposited on the collector of the measuring electrode 20 contains, per 1 ml of 10 mM phosphate buffer adjusted to pH 6.8: 1 to 1000 mg of carbon powder, preferably 1 to 100 mg or better about 10 mg; 1 to 2000 IU of glucose oxidase per mg of carbon powder, preferably 10 to 300 IU or better 100 IU, and 1 to 10000 µmol of mediator per mg of carbon powder, preferably 10 to 300 µmol or better 50 µmol. This mixture is deposited in a proportion of 10 to 300 µliter/$cm^2$ of active surface, preferably 30 to 150 µliter/$cm^2$ or better 70 µliter/$cm^2$.

In the finished, dried sensor, it can therefore be considered that the mixture 38 contains 1 to 2000 IU of glucose oxidase per mg of carbon powder, preferably 10 to 3000 [sic] IU or better 100 IU and 1 to 10000 µmol of mediator per mg of carbon powder, preferably 10 to 300 µmol or better 50 µmol.

The sensor according to the invention, equipped with the aforesaid mediators, has a certain number of properties that vary as a function of the ligands used and of the substituents on these ligands.

Several experiments were carried out which prove the performances and the efficacy of these new mediators and which yield the conditions for optimizing the various elements constituting the measuring electrode. These experiments are described in the following.

Experiment 1:

Measurements of different mediators by cyclic voltametry.

a) Measurements made with the tris(4,4'-dimethoxy-2,2'-bipyridine) complex of osmium.

The aforesaid complex was tested by direct-current cyclic voltametry, in order to determine on the one hand its normal oxidation-reduction potential E° and on the other hand the rate constant k. This constant k corresponds to the reaction of electron transfer from GOD to the mediator. The cyclic voltametry consists in placing a working electrode, a counterelectrode and a reference electrode in the solution to be analyzed, then in running a constant-speed scan of the potential of the working electrode between two terminals and measuring the obtained current intensity. The curves of Figures 5 and 6 represent the results obtained by this method. These experiments were carried out with a glassy carbon working electrode, a calomel reference electrode, a platinum counterelectrode and an electrochemical cell of 5 to 20 ml capacity. The measurements were performed in phosphate buffer (PBS) (100 mM NaCl, 10 mM $NaH_2PO_4$) adjusted to pH 7.4; 0.1 mM EDTA (ethylenediaminetetraacetic acid); 0.01 mM PMSF (phenylmethylsulfonate fluoride) and with a concentration of the aforesaid complex of $5.10^{-4}$ M. Different potential scanning speeds were used: 5, 10, 25, 50 and 100 $mV.s^{-1}$. The curves of Figure 5 as well as a value of E° of 225 mV were obtained. The addition of saturated glucose solution did not have any effect on the curves of Figure 5, which is normal, since glucose oxidase (GOD) was not present.

On the other hand, the addition of GOD (in a quantity greater than $5.10^{-8}$ M and preferably $4.10^{-6}$ M) permits the curves of Figure 6 to be obtained, with their characteristic "catalytic wave" form. In this Figure 6, the following potential scanning speeds were used: 10, 25, 50 and 100 $mV.s^{-1}$.

A first irreversible reaction occurs (with a constant k):

$$\text{mediator}_{(\text{oxidized})} + \text{GOD}_{(\text{reduced})} \xrightarrow{k} \text{mediator}_{(\text{reduced})} + \text{GOD}_{(\text{oxidized})}$$

and is followed by a second reaction:

$$\text{mediator}_{(\text{reduced})} + e^- \rightarrow \text{mediator}_{(\text{oxidized})}$$

which is electrochemically reversible and extremely rapid.

The mediator brings about electrochemically reversible transfer of an electron to the current collectors described in the foregoing.

During the first reaction, the second-order rate constant k can be measured. For the complex studied here, it is found that $k = 2.5.10^6 \pm 0.5\ M^{-1}.s^{-1}$.

b) Measurements made with other complexes.

Experiments similar to those just described were carried out for other complexes. Table 1 presents the values of the found rate constants k and of the normal oxidation-reduction potentials E° in mV relative to a calomel reference electrode (SCE).

TABLE 1

| | Complexes | E° (mV/SCE) | k ($M^{-1}.s^{-1}$) |
|---|---|---|---|
| 1 | tris(4,4'-dimethoxy-2,2'-bipyridine) complex of osmium | 225 | $2.5.10^6$ |
| 2 | bis(4,4'-dimethoxy-2,2'-bipyridine)-mono-(4,4'-dimethyl-2,2'-bipyridine) complex of osmium | 340 | $2.10^6$ |

| | | | | |
|---|---|---|---|---|
| 3 | bis(4,4'-dimethyl-2,2'-bipyridine)-mono-(4,4'-dimethoxy-2,2'-bipyridine) complex of osmium | | 390 | N.D. |
| 4 | mono-(4,4'-dimethoxy-2,2'-bipyridine)-mono-(4,4'-dihydroxy-2,2'-bipyridine)-mono-(4,4'-dimethyl-2,2'-bipyridine) complex of osmium | | | |
| | | pH < 4.5 | 340 | N.D. |
| | | pH > 4.5 | 190 | $2.10^5$ |
| 5 | tris(4,4'-dimethyl-2,2'-bipyridine) complex of osmium | | 425 | $1.5.10^6$ |
| 6 | tris(4,4'-dihydroxy-2,2'-bipyridine) complex of osmium | | - 1000 | - 0 |
| 7 | tris(4,4'-diamino-2,2'-bipyridine) complex of ruthenium | | 170 | $1.6.10^6$ |
| 8 | tris(4,4'-diamino-2,2'-bipyridine) complex of iron | | 70 | $1.4.10^5$ |

N.D. denotes not determined.

This Table 1 shows that the family of mediators has a very broad range of redox potentials, varying between - 1000 mV and + 425 mV (relative to a calomel reference electrode (SCE)). The lower limit of this range is much lower than all the redox potentials of mediators heretofore described in the literature. In addition, this range of potentials is also much broader than that obtained with the ferrocenes family. This is due to the large number of substituents that can be used and to the greater number of possible combinations of substituents.

The second-order constant $k_f$ corresponding to the rate constant of the oxidation-reduction reaction between the enzyme and the mediator according to the invention is much faster than with the other mediators known to this date, and is faster than with oxygen. In fact, oxygen has a constant k of only $1.5.10^6$ $M^{-1}.s^{-1}$. This limits the problems described in the foregoing of competition of oxygen with the mediator during the reaction of electron transfer from the GOD. Similarly, other competitive reactions that take place much more slowly do not influence the result of the measuring apparatus.

Consequently, complexes 1 and 2 have been selected as mediators for the glucose sensors in order to illustrate the continuation of the description, since these complexes have the most favorable characteristics, in other words a high constant k and simultaneously a low normal oxidation-reduction potential $E^\circ$, which is nevertheless higher than - 300 mV, which corresponds to the normal potential of the $FAD/FADH_2$ group of GOD.

Experiment 2:

Optimization of the different components of the mixture of the measuring electrode.

Attempts were made to determine the respective optimum quantities of the different constituents of the mixture deposited on the collector of the measuring electrode.

This was achieved by making a mixture 38 containing one of the two preferred complexes cited in the foregoing, carbon powder, immobilized glucose oxidase and, as additive, bovine serum albumin and glutaraldehyde, then depositing a quantity of 70 µliter of this mixture per $cm^2$ on the current-collector part 37 of the electrically conductive strip 36 in order to form a measuring electrode 20. Different types of measuring electrodes were then made by gradually varying one of the components of the mixture while keeping the others constant.

The different sensors prepared in this way were used for potentiostatic measurements at a potential of 300 mV, in multiple blood

samples containing various quantities of glucose. The results are illustrated in the following.

a) Optimization of the quantity of carbon powder.

Several different types of sensors were made (although the choice was made to present only three thereof) by mixing, in 3 ml of phosphate buffer (PBS), a constant quantity of GOD (36.9 mg), a constant quantity of bis(4,4'-dimethoxy-2,2'-bipyridine)-mono-(4,4'-dimethyl-2,2'-bipyridine) complex of osmium (3.0 mg), used as mediator, a constant quantity of 25% glutaraldehyde (25 µliter), a constant quantity of 15% bovine serum albumin (290 µliter) and respectively 25, 50 or 250 mg of carbon powder.

The phosphate buffer (PBS) used here and in the experiments mentioned below is a 10 mM buffer adjusted to pH 6.8.

These three types of sensor were then tested in a physiological solution containing different quantities of glucose (between 0 and 20 mM of glucose), and the current density obtained after 30 seconds ($D_{30}$) was measured. The physiological solution consisted of 115 mM NaCl, 25 mM KCl, 5 mM $K_2HPO_4.3H_2O$ and 0.5 mM $KH_2PO_4$.

The results obtained are illustrated in Figure 7, where the lines a, b, c correspond respectively to the results observed with sensors containing 25, 50 and 250 mg of carbon in 3 ml of phosphate buffer (PBS), i.e., approximate concentrations of 8, 17 and 83 mg per ml. Here again, it must be noted that many more measurements were made, but that the choice was made to represent only the lines a, b, c.

The slope (m) of all the lines representing the totality of the measurements performed was then calculated, and these values were plotted in Figure 8 (curve $C_1$), where the abscissa represents the quantity of carbon in mg per ml of phosphate buffer (PBS). Similarly, the ordinate of these lines at the origin was calculated, and these values were also plotted in Figure 8 (curve $C_2$). The ordinate at the origin corresponds to the value of the point of intersection of a line of Figure 7 with the ordinate, i.e., to the value of the residual current.

Obviously curve $C_1$ is substantially horizontal between 17 and 83 mg of carbon, meaning that the quantity of carbon has little influence on the results of the sensor between these two values. Nevertheless, since a thin layer of carbon has better mechanical and diffusion properties, it is preferred to use the least possible quantity of carbon. In addition, the ordinate of line a at the origin obviously has the lowest value (8 mg of carbon per ml), meaning that the smallest residual current is obtained in this case.

Consequently, it is preferred to use about 10 mg of carbon per ml of phosphate buffer (PBS).

b) Optimization of the quantity of enzyme (GOD).

Several different types of sensors were made (although the choice was made to present only three thereof) by mixing, in 3 ml of phosphate buffer (PBS), a constant quantity of carbon (25 mg), a constant quantity (3.0 mg) of the same mediator as that of paragraph a), constant quantities of 25% glutaraldehyde (25 µliter) and 15% bovine serum albumin (290 µliter) and quantities of glucose oxidase (GOD) of respectively 2175, 4375 and 8750 IU, i.e., GOD concentrations of respectively 87, 175 and 350 IU of glucose oxidase (GOD) per mg of carbon. [sic - redundancies in original]

The same series of measurements and calculations were then carried out as in paragraph a). The lines a, b, c of Figure 9 correspond respectively to the results observed with the sensors containing 87, 175 and 350 IU of glucose oxidase per mg of carbon powder. The curves $C_1$ and $C_2$ of Figure 10 represent respectively the slope (m) and the ordinate at the origin. The abscissa of Figure 10 expresses the quantity of GOD in IU per mg of carbon powder.

Obviously the curve $C_1$ is substantially horizontal between 75 and 350 IU of GOD per mg of carbon powder, meaning that the quantity of GOD has little influence on the results between these two values. Furthermore, the ordinate of line a at the origin has the lowest value, meaning that the smallest residual current is obtained in this case.

Consequently, it is preferred to use about 100 IU of GOD per mg of carbon powder.

c) Optimization of the quantity of mediator

Three different types of sensors were made by mixing, in 3 ml of phosphate buffer (PBS), a constant quantity of carbon (25 mg), a constant quantity of GOD (36.9 mg), constant quantities of 25% glutaraldehyde (25 µliter) and 15% bovine serum albumin (290 µliter) and respectively 825, 1675 and 3325 µmol of bis(4,4'-dimethoxy-2,2'-bipyridine)-mono-(4,4'-dimethyl-2,2'-bipyridine) complex of osmium, i.e., mediator concentrations of 33, 67 and 133 µmol per mg of carbon powder.

The same series of measurements and calculations were then carried out as in paragraph a). The lines a, b, c of Figure 11 correspond respectively to the results observed with 33, 67 and 133 µmol of this complex per mg of carbon. The curves $C_1$ and $C_2$ of Figure 12 represent respectively the slope (m) and the ordinate at the origin. The abscissa of Figure 12 expresses the quantity of mediator in µmol per mg of carbon powder.

Obviously the curves $C_1$ and $C_2$ are substantially horizontal. For mediator values below 50 µmol, the measurements necessarily had to be made at a potential above 300 mV. Since it is preferred to work at the lowest possible potential, it is therefore preferred to use about 50 µmol of mediator per mg of carbon powder.

The optimization tests performed for the bis(4,4'-dimethoxy-2,2'-bipyridine)-mono-(4,4'-dimethyl-2,2'-bipyridine) complex of osmium are also valid for the tris(4,4'-dimethoxy-2,2'-bipyridine) complex of osmium.

Experiment 3:

Calibration of the sensor in blood and in buffer.

The curves of Figure 13 illustrate the potentiostatic measurements with sensors having the two preferred complexes of the invention as mediator, which measurements were made by varying the glucose concentration in samples of blood or of phosphate buffer (PBS). The measurements were made at 300 mV, and the current density D20 was read after 20 seconds.

The curves $C_1$ and $C_3$ correspond respectively to the measurements made in the phosphate buffer and in blood with a sensor using the tris(4,4'-dimethoxy-2,2'-bipyridine) complex of osmium, while the curves $C_2$ and $C_4$ correspond respectively to the measurements made in the phosphate buffer and in blood with a sensor using the bis(4,4'-dimethoxy-2,2'-bipyridine)-mono-(4,4'-dimethyl-2,2'-bipyridine) complex of osmium.

As can be seen in Figure 13, the different curves are linear and have a sufficiently large slope up to values of 20 mM of glucose. Consequently, in a patient in whom the physiological values of glucose can typically vary from 3 to 20 mM, the sensor according to the invention can be used reliably, since a slight variation of the glucose concentration brings about a sufficient corresponding variation of the measured current density.

The differences observed between the measurements made in the buffer (PBS) and in whole blood are due to the same phenomenon as that described for plasma and whole blood, among other by Fogh-Andersen, N. et al. (1990), Clin. Chim. Acta 189, 33-38. This difference is due mainly to the volume occupied by the proteins in whole blood.

Experiment 4:

Influence of hematocrit on the results supplied by the sensor.

The curves of Figure 14 illustrate the variations of current density ($D_{30}$) obtained after 30 seconds as a function of the glucose concentration in artificially reconstituted human blood. The blood samples were prepared in the following manner. The plasma and blood cells were separated by centrifuging at 3000 rpm for 15 minutes at 4°C. The blood was then reconstituted so as to obtain various values of the hematocrit (0.35, 0.50 and 0.60), and certain quantities of glucose were added to these samples. The glucose concentration was measured by using a calibrated laboratory apparatus, for example the model 23A apparatus (available from Yellow Springs Instrument, Yellow Springs, OHIO). The potentiostatic measurements were performed at 300 mV with sensors containing the bis(4,4'-dimethoxy-2,2'-bipyridine)-mono-(4,4'-dimethyl-2,2'-bipyridine) complex of osmium as mediator. The measurements of current density were made after 30 seconds.

The curves $C_1$, $C_2$ and $C_3$ correspond respectively to samples containing 35% cells and 65% plasma, 50% cells and 50% plasma, and 60% cells and 40% plasma.

The curve $C_2$ corresponds to a normal hematocrit. It can be seen that curve $C_3$ (hematocrit 0.60), which corresponds to a high hematocrit, practically does not differ from curve $C_2$.

On the other hand, curve $C_1$ (hematocrit 0.35), which corresponds to the hematocrit of an anemic person, differs from curve $C_2$.

Consequently, the sensor according to the invention yields reliable results in a patient having a high hematocrit, but less reliable results in a person with anemia.

Experiment 5:

Influence of pH on the mediator activity of the tris(4,4'-dimethoxy-2,2'-bipyridine) complex of osmium and of the bis(4,4'-dimethoxy-2,2'-bipyridine)-mono-(4,4'-dimethyl-2,2'-bipyridine) complex of osmium

These two complexes were mixed in a phosphate buffer solution (PBS), after which the pH was varied and the normal oxidation-reduction potential E° was measured.

A stable potential E° was observed for a pH between 1 and 12. This potential E° was + 225 mV for the first complex and + 340 mV for the second. Since the pH of human blood is about 7.4 in practice, slight variations of blood pH do not affect the result for glycemia measured by the sensor according to the invention.

Experiment 6:

Influence of the presence of certain drugs on the results supplied by the sensor.

Finally, a last series of experiments was carried out in order to check whether the results supplied by this sensor could be influenced by the presence of drugs existing in the blood at the time of the measurement. In practice, it is entirely possible that a patient would ingest drugs such as aspirin or vitamin C before making a measurement of glycemia.

Consequently, tests were made of the possible influence of acetylsalicylic acid, acetaminophenol and ascorbic acid on the results supplied by the sensor according to the invention.

The experiments were carried out with a sensor using the tris(4,4'-dimethoxy-2,2'-bipyridine) complex of osmium as mediator.

The potentiostatic measurements were performed at 300 mV. The current density ($D_{30}$) was read after 30 seconds. The different curves represent the variations of current density as a function of glucose

concentration when different quantities of each of the drugs being tested are present in a sample of physiological solution.

The results obtained are the following:

- Acetaminophenol:

Figure 15 illustrates the curves obtained. The curves $C_1$, $C_2$ (dashed) and $C_3$ correspond respectively to acetaminophenol concentrations of 0, 50 and 500 µM.

The value of 50 µM corresponds to what would be found in a patient who has absorbed acetaminophenol in a normal dosage, whereas the value of 500 µM corresponds to an overdose. Obviously the presence of this drug has hardly any influence on the results supplied by this sensor between glucose concentrations of 4 and 10 mM (which correspond substantially to physiological values), since all curves are substantially superposed.

- Ascorbic acid:

Figure 16 illustrates the curves obtained. The curves $C_1$, $C_2$ and $C_3$ correspond respectively to concentrations of 0, 100 and 1000 µM of ascorbic acid per ml [sic] of blood.

The value of 100 µM (curve $C_2$) corresponds to the values that would be found in a patient who has absorbed vitamin C in a normal dosage, whereas the value of 1000 µM (curve $C_3$) corresponds to an excess of ascorbic acid.

It is therefore seen that all values of the glucose concentration are increased relative to the normal levels during the presence of excess ascorbic acid (curve $C_3$). In contrast, curve $C_2$ is substantially identical to $C_1$, and so it is seen that the presence of ascorbic acid in physiological values does not affect the results supplied by the sensor.

Acetylsalicylic acid

It was not considered necessary to present a figure illustrating the obtained results obtained, since it was found that a quantity of acetylsalicylic acid as high as 25 mM yielded straight lines substantially identical to the line corresponding to a quantity of 0 mM of acetylsalicylic acid. Consequently, it was deduced that the presence of acetylsalicylic acid does not affect the results supplied by the sensor.

CLAIMS

1. A sensor for measuring the quantity of a component in solution, comprising:

- at least one measuring electrode (20) and one reference electrode (22), electrically insulated from each other and intended to come into contact with the said solution, the said electrodes (20, 22) respectively containing electrical contacts (34; 26) adapted to be connected to a device (4) for processing the signal supplied by the said sensor,

- the measuring electrode (20) containing at least one current collector (37), connected electrically to one of the electrical contacts (34) and covered with a mixture (38) containing at least one oxidation-reduction enzyme that is specific for the said component and at least one mediator that transfers the electrons between the said enzyme and the said current collector, characterized in that the mediator is chosen from among the complexes of a transition metal with at least one bipyridine, terpyridine or phenanthroline ligand substituted by at least one electron donor group.

2. A sensor according to claim 1, characterized in that the mixture (38) of the measuring electrode (20) also contains an active conductive material and in that the mediator transfers the electrons between the enzyme and this active conductive material.

3. A sensor according to claim 1, characterized in that the transition metal is chosen from among iron, ruthenium, osmium or vanadium.

4. A sensor according to claim 1, characterized in that the electron donor group is chosen from among the OH group, an alkoxy group, an aryloxy group or a primary, secondary or tertiary amine group.

5. A sensor according to claim 1, characterized in that the oxidation-reduction enzyme is chosen from among the oxidases or the flavoproteins.

6. A sensor according to claim 5 for measuring the quantity of glucose present in the solution, characterized in that the enzyme is glucose oxidase.

7. A sensor according to claim 1, characterized in that the mediator is chosen from among the tris(4,4'-dimethoxy-2,2'-bipyridine) complex of osmium or the bis(4,4'-dimethoxy-2,2'-bipyridine)-mono-(4,4'-dimethyl-2,2'-bipyridine) complex of osmium.

8. A sensor according to claim 2, characterized in that the active conductive material is a powder of a material chosen from among carbon, gold, platinum, palladium or a conductive metal oxide.

9. A sensor according to claim 2, characterized in that the active conductive material is a film of a conductive polymer.

10. A sensor according to claim 1 or 2, characterized in that the mixture (38) of the measuring electrode (20) contains an additive that forms a network for immobilization of the enzyme of the mediator and/or of the active conductive material on the surface of the collector (37) of the measuring electrode (20).

11. A sensor according to claim 10, characterized in that the additive is chosen from among bovine serum albumin, glutaraldehyde, carbodiimide or water-soluble polymers.

12. A sensor according to claims 6, 7 and 8, characterized in that the mixture (38) deposited on the collector of the measuring electrode (20) contains between 1 and 2000 IU of glucose oxidase per mg of carbon powder and between 1 and 10000 µmol of mediator per mg of carbon powder.

13. A sensor according to claim 12, characterized in that the mixture (38) deposited on the collector of the measuring electrode (20) contains between 10 and 300 IU of glucose oxidase per mg of carbon powder and between 10 and 300 µmol of mediator per mg of carbon powder.

14. A sensor according to claim 13, characterized in that the mixture (38) deposited on the collector of the measuring electrode (20) contains above 100 IU of glucose oxidase per mg of carbon powder and about 50 µmol of mediator per mg of carbon powder.

15. An assembly, characterized in that it consists of a sensor according to any one of claims 1 to 14 and of a device for processing the signal supplied by the said sensor, this device containing at least two electrical contacts designed to be connected to at least two electrodes of the said sensor, an ammeter and means for indicating the results.

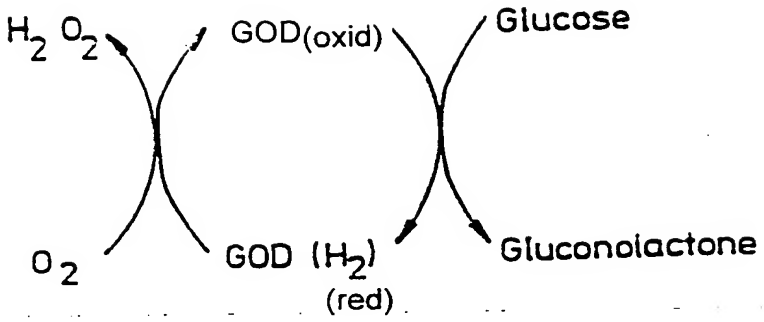

$H_2O_2$
GOD(oxid)
Glucose
$O_2$
GOD ($H_2$)
(red)
Gluconolactone

Fig.1

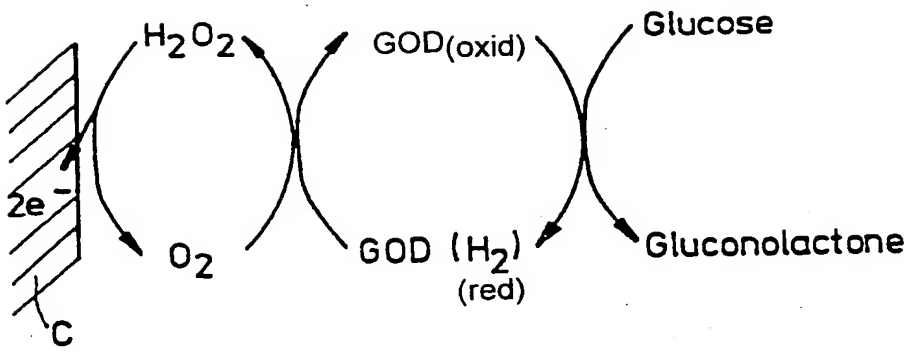

$H_2O_2$
GOD(oxid)
Glucose
$2e^-$
$O_2$
GOD ($H_2$)
(red)
Gluconolactone
C

Fig. 2

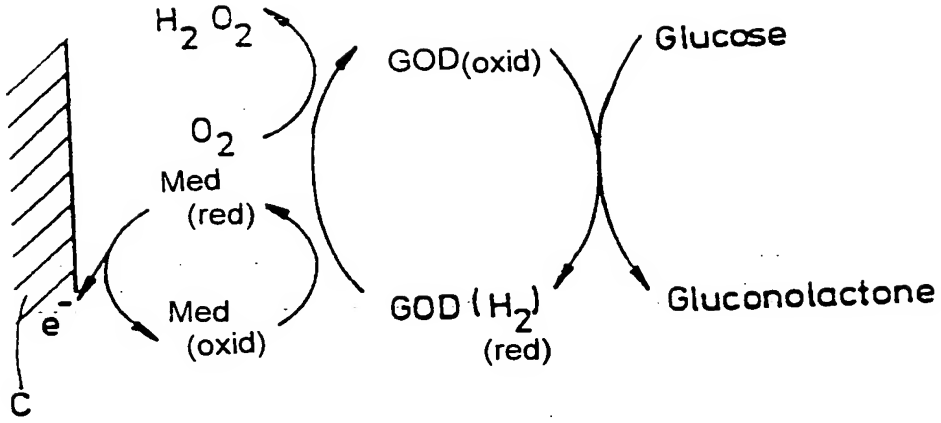

$H_2O_2$
$O_2$
Med
(red)
Med
(oxid)
$e^-$
C
GOD(oxid)
GOD ($H_2$)
(red)
Glucose
Gluconolactone

Fig. 3

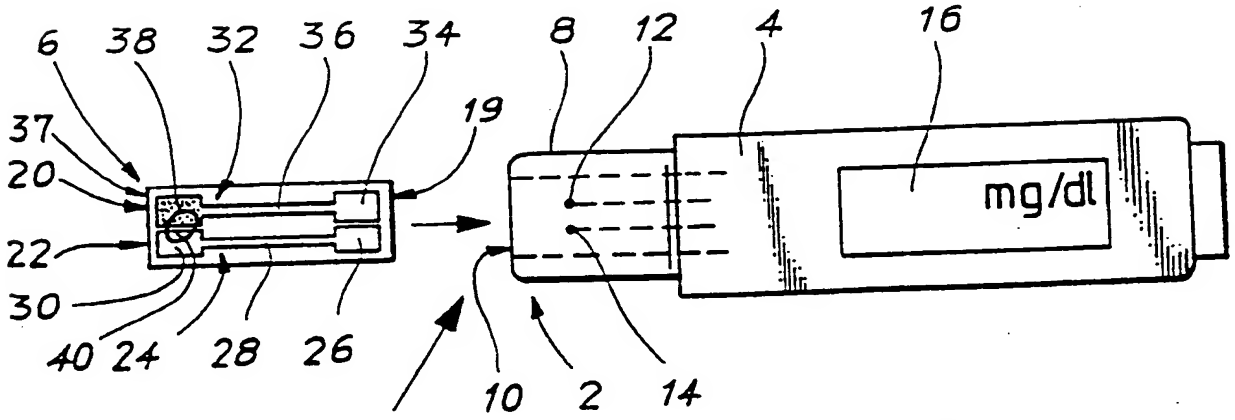
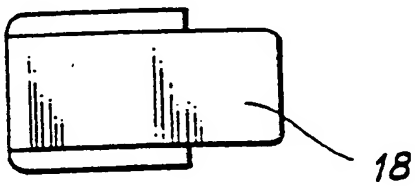

6
38
32
36
34
8
12
4
16
37
20
19
22
30
40
24
28
26
10
2
14
mg/dl

Fig. 4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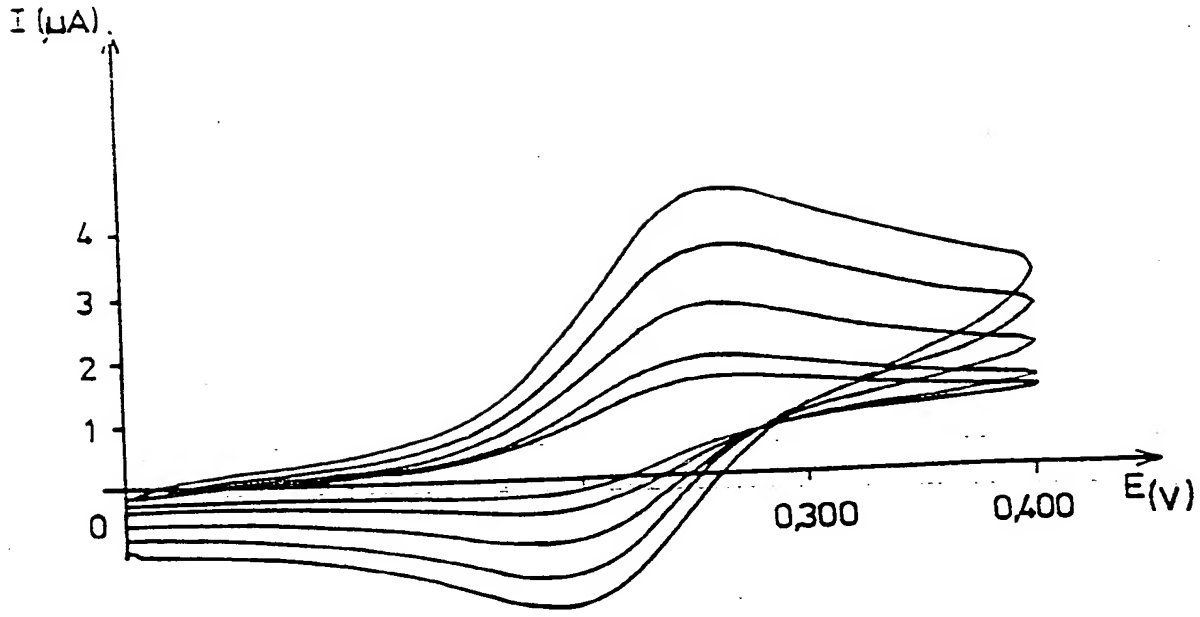
I (µA)
4
3
2
1
0
0,300
0,400
E(V)

Fig. 5

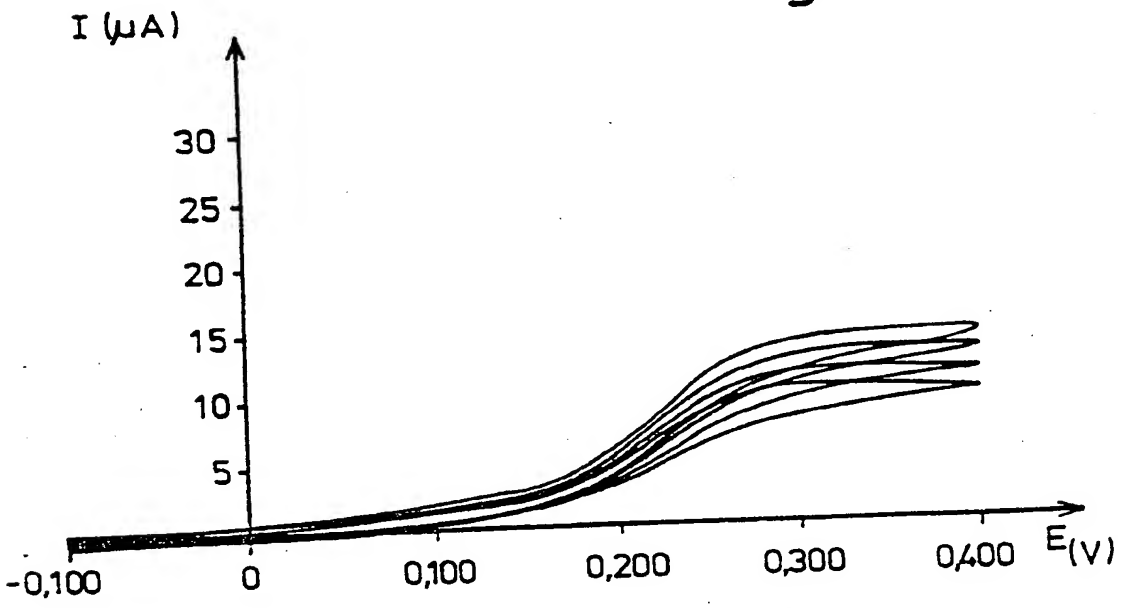
I (µA)
30
25
20
15
10
5
-0,100
0
0,100
0,200
0,300
0,400
E(V)

Fig. 6

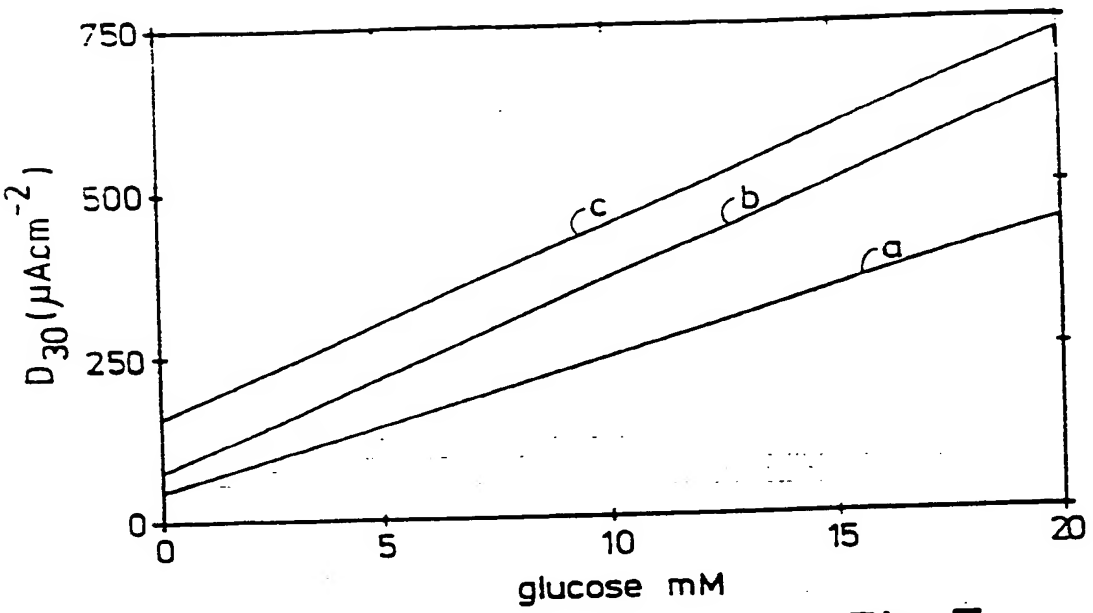
750
500
250
0
$D_{30}$ ($\mu A cm^{-2}$)
c
b
a
0
5
10
15
20
glucose mM

Fig.7

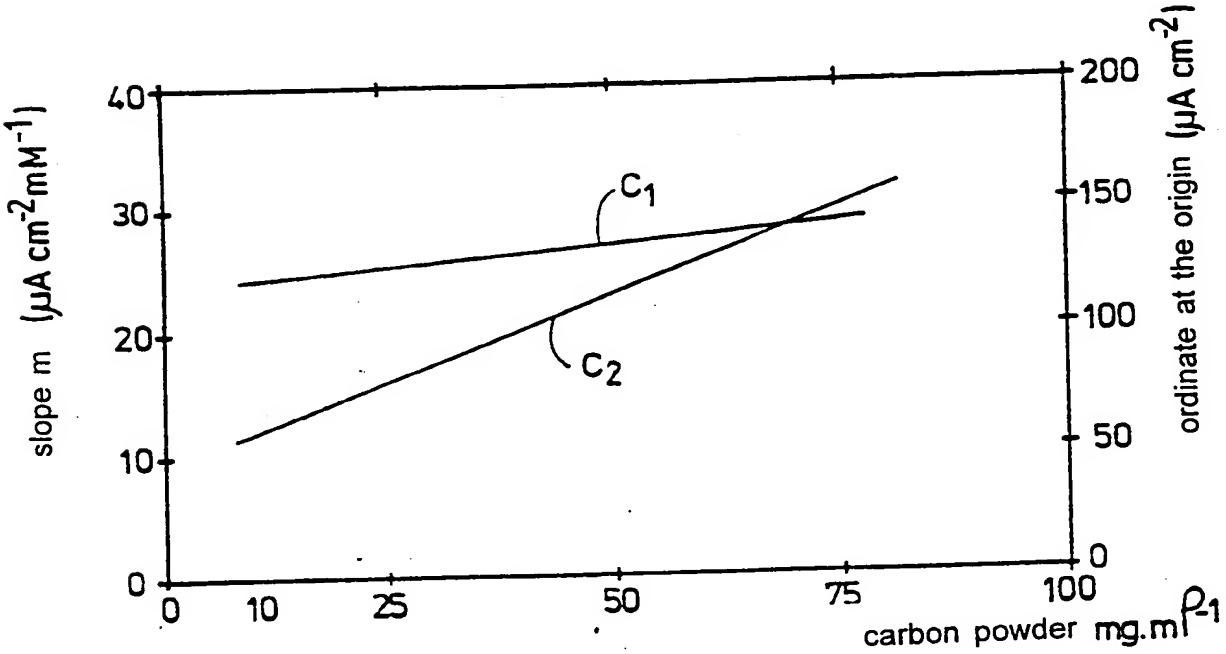
40
30
20
10
0
slope m ($\mu A\, cm^{-2} mM^{-1}$)
$C_1$
$C_2$
200
150
100
50
0
ordinate at the origin ($\mu A\, cm^{-2}$)
0
10
25
50
75
100
carbon powder mg.ml$^{-1}$

Fig.8

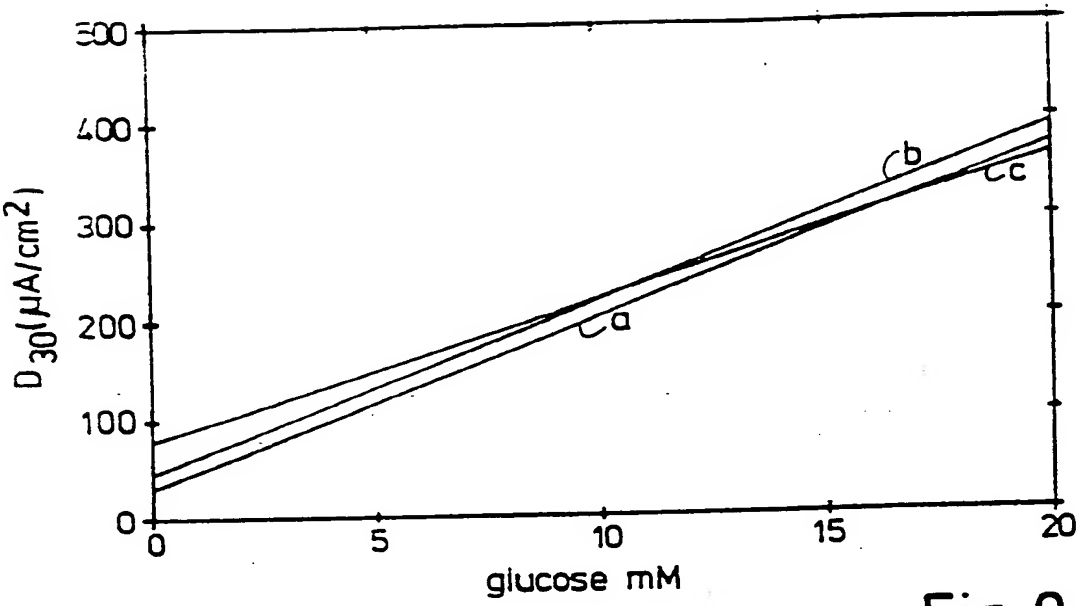
500
400
300
200
100
0
$D_{30}(\mu A/cm^2)$
0
5
10
15
20
glucose mM
a
b
c

Fig.9

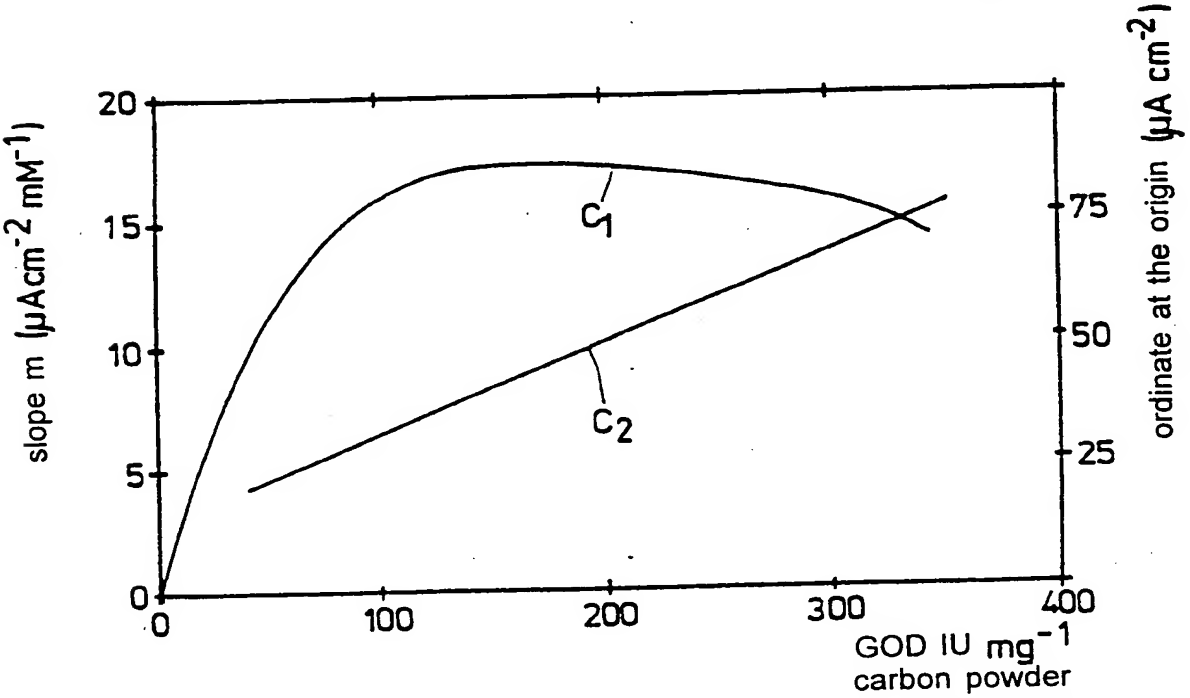
20
15
10
5
0
slope m ($\mu A cm^{-2} mM^{-1}$)
0
100
200
300
400
GOD IU $mg^{-1}$ carbon powder
75
50
25
ordinate at the origin ($\mu A\ cm^{-2}$)
$C_1$
$C_2$

Fig.10

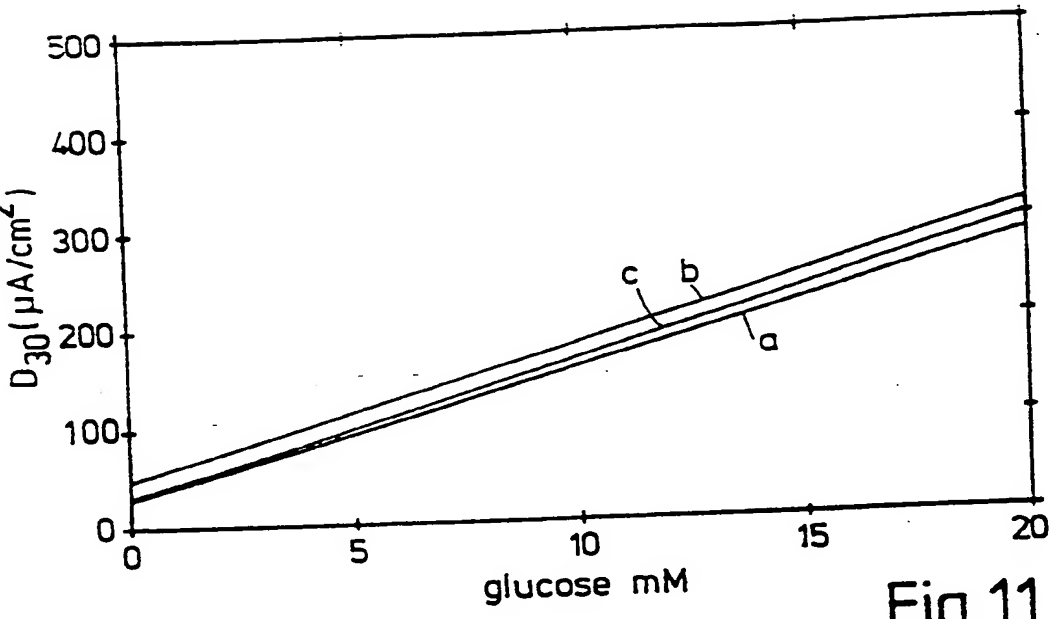
500
400
300
200
100
0
$D_{30}$ (µA/cm²)
c
b
a
0
5
10
15
20
glucose mM

Fig.11

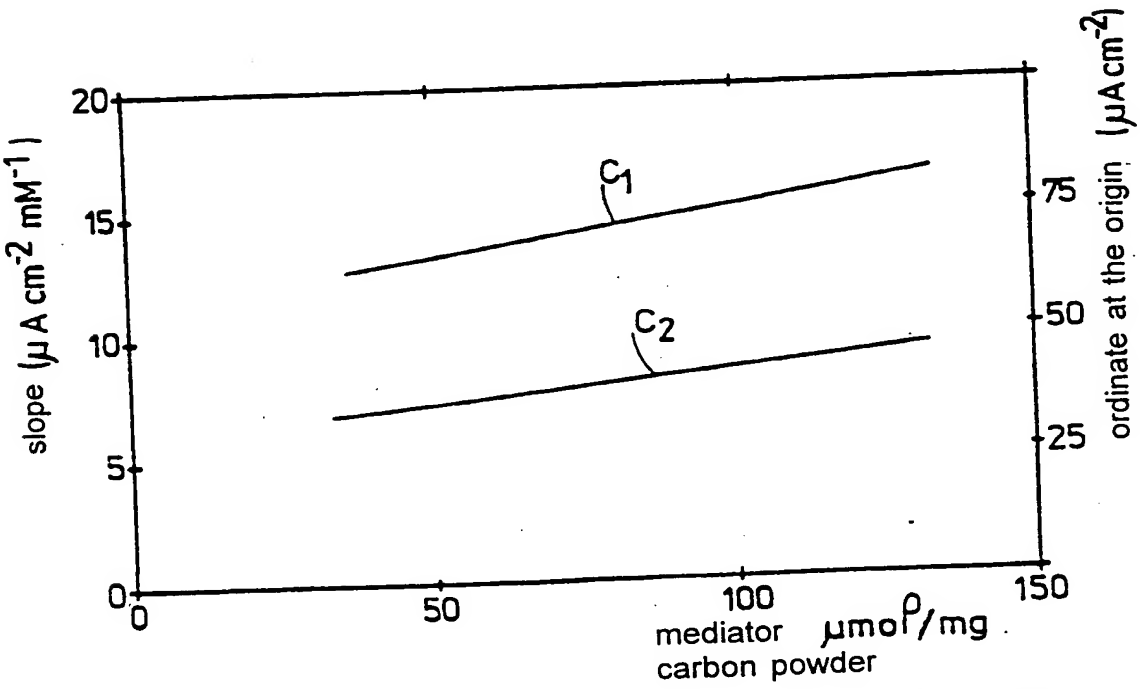
20
15
10
5
0
slope (µA cm⁻² mM⁻¹)
C1
C2
75
50
25
ordinate at the origin (µA cm⁻²)
0
50
100
150
mediator µmol/mg
carbon powder

Fig.12

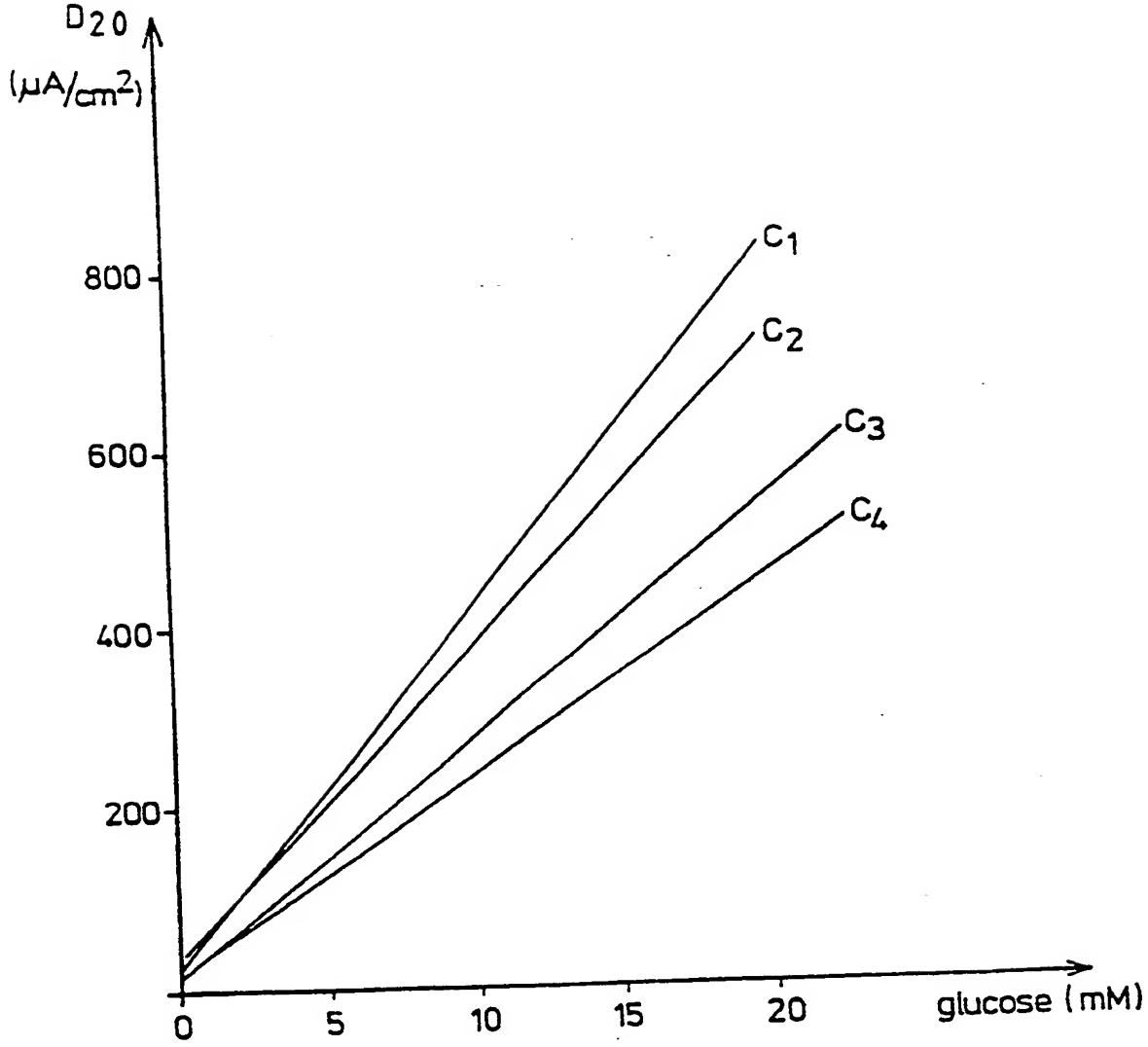


Fig. 13

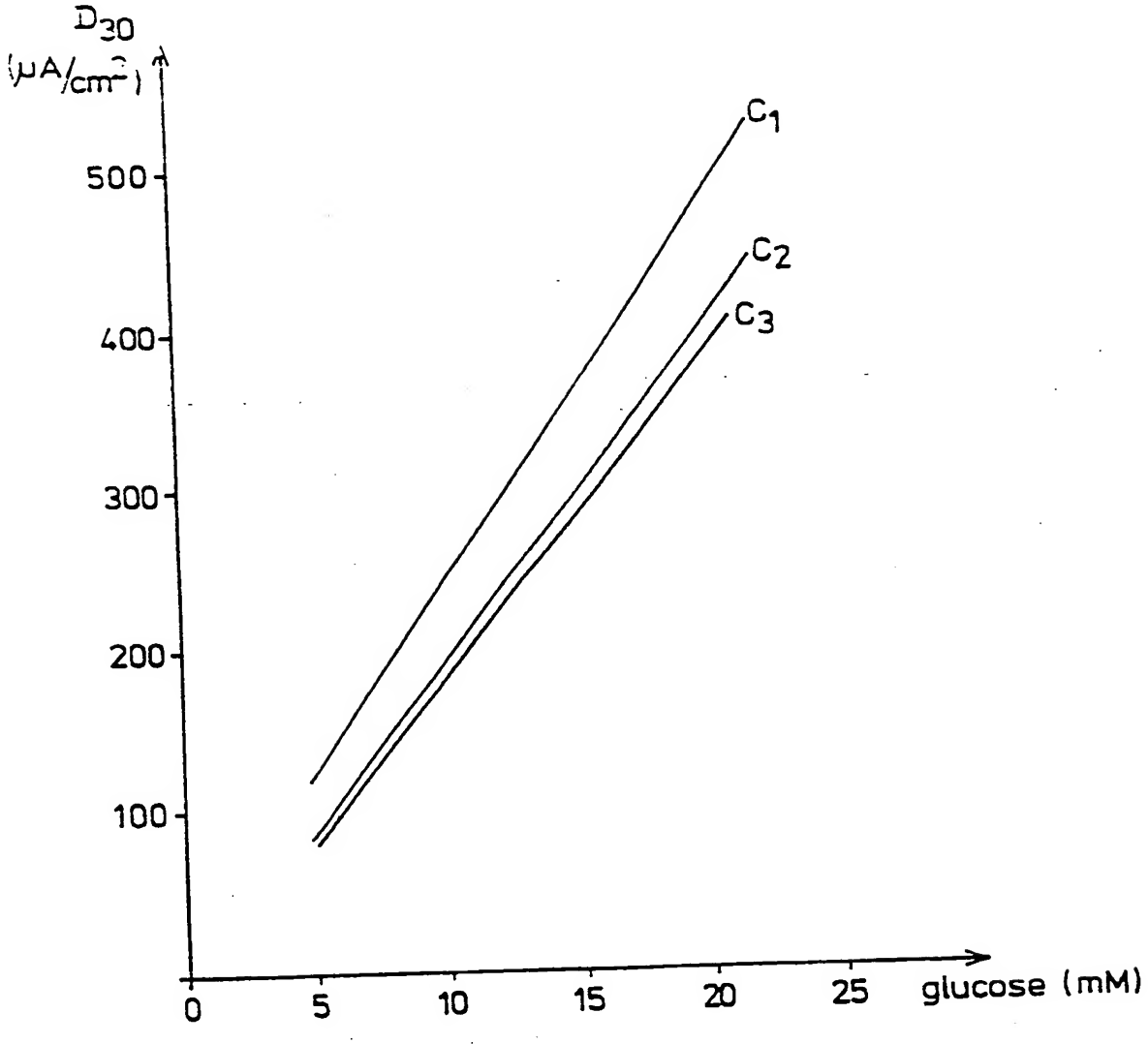


Fig.14

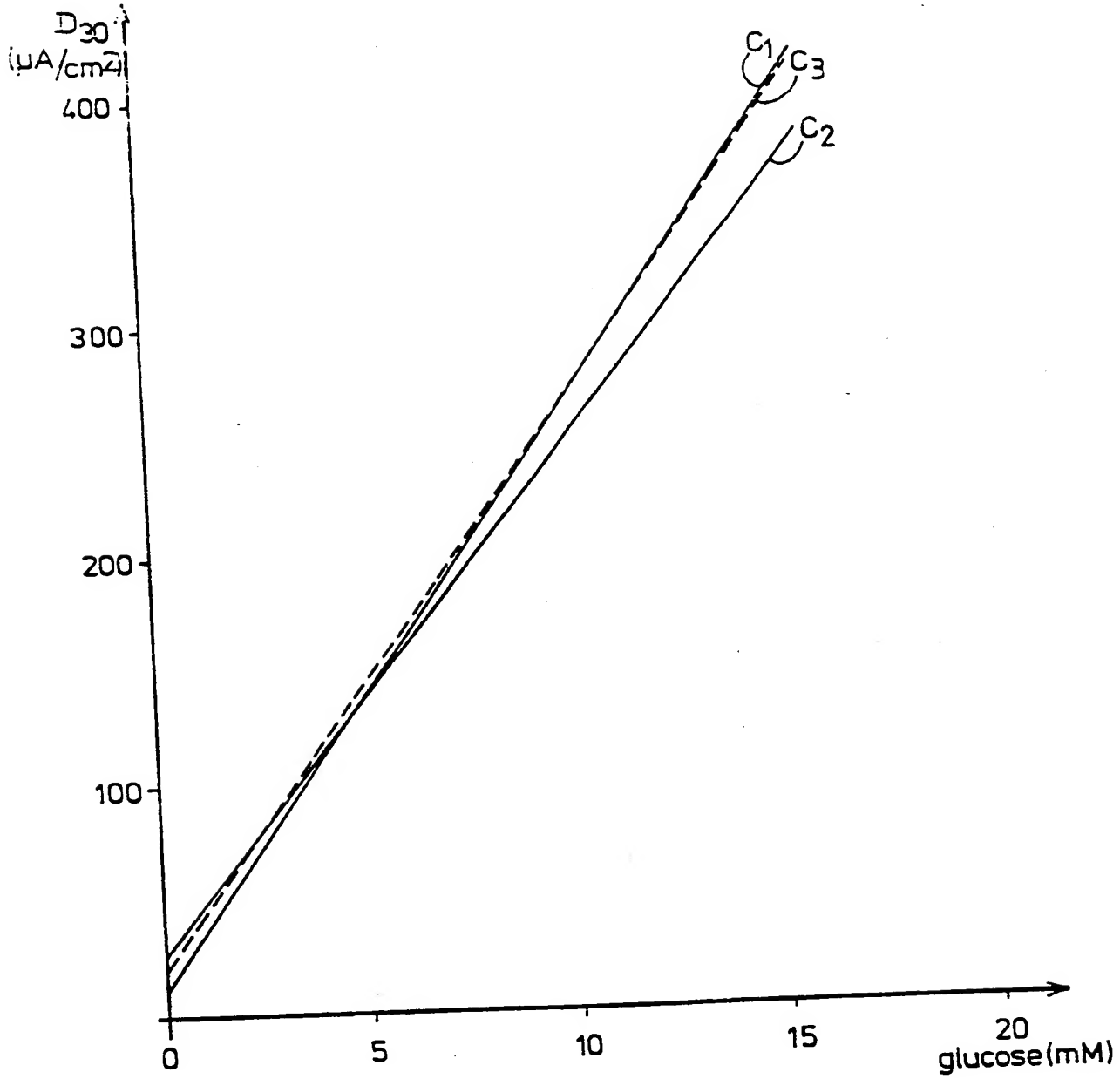


Fig.15

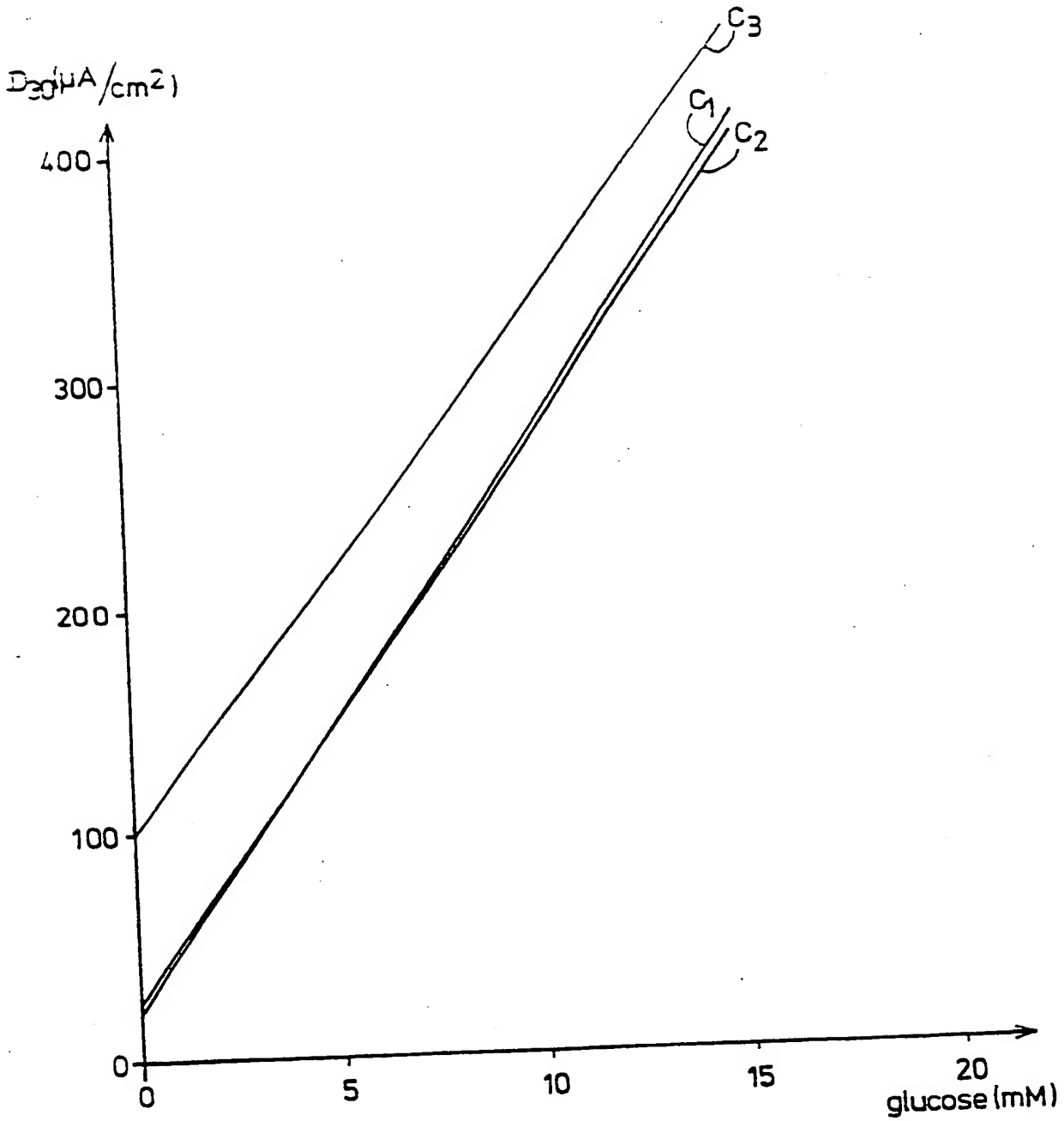


Fig. 16

# INTERNATIONAL SEARCH REPORT

International Application No PCT/CH 92/00034

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C12Q1/00; C12Q1/54

## II. FIELDS SEARCHED

Minimum Documentation Searched 7

| Classification System | Classification Symbols |
|---|---|
| Int.Cl.5 | C12Q; G01N |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched 8

## III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

| Category * | Citation of Document, 11 with indication, where appropriate, of the relevant passages 12 | Relevant to Claim No. 13 |
|---|---|---|
| Y | EP, A, 0 096 288 (BASF) 21 December 1983<br>see page 2, line 27 - line 36;<br>examples page, 5<br>--- | 1-14 |
| Y | ANGEWENDTE CHEMIE<br>vol. 102, no. 1, 1990, WEINHEIM BRD<br>pages 109 - 111;<br>M.V. PISHKO ET AL: 'Direct electron exchange between graphite electrodes and an adsorbed complex of glucose oxidase and an Os-containing redox polymer'<br>*whole document*<br>--- | 1-14 |
| Y | JOURNAL OF ELECTROANALYTICAL CHEMISTRY<br>vol. 286, 1990, LAUSANNE CH<br>pages 75 - 87;<br>J.P. COLLIN ET AL: 'Anodic electropolymerization of films of polypyrrole functionalized with metal terpyridyl redox centers'<br>*whole document*<br>---<br>-/-- | 1-14 |

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 29 May 1992 (29.05.92) | 9 June 1992 (09.06.92) |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| A | ANALYTICAL CHEMISTRY<br>vol. 54, 1982, WASHINGTON DC USA<br>pages 1377 - 1383;<br>J.M.JOHNSON ET AL:'Potential-dependent enzymatic activity in an enzyme thin-layer cell' *whole document * | 1-14 |
| A | BIOCHEMISTRY<br>vol. 24, no. 7, 26 March 1985 WASHINGTON DC USA Pages 1579 - 1585;<br>J.M. JOHNSON: 'redox activation of galactose oxidase:thin layer electrochemical study' *whole document* | 1-14 |
| A | WO, A, 8 505 119 (STIFTUNG R. E.) 21 November 1985<br>see the whole document | 1-14 |
| A | PATENT ABSTRACTS OF JAPAN<br>vol. 12, no. 96 (C-484) (2943) 29 March 1988<br>& JP, A, 62 228 274 (MITSUBISHI CHEMICAL INDUSTRIES LTD) 7 October 1987<br>see abstract | 1-14 |